*ALKALINE pH DEPENDENCE OF δ-CHYMOTRYPSIN-CATALYZED HYDROLYSIS OF SPECIFIC SUBSTRATES**

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Abstract.—K_m (app) and k_{cat} values for the δ-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester and *N*-furylacryloyl-L-tryptophanamide were measured as a function of pH and ionic strength. The K_m (app) values do not increase considerably above pH 9 for δ-chymotrypsin, as is the case with α-chymotrypsin. The observed kinetic difference between both enzymes at high pH suggests that the reversible inactivation of α-chymotrypsin at alkaline pH may involve the participation of tyrosine 146 or alanine 149 since both residues are present as chain termini in α-chymotrypsin but not in δ-chymotrypsin.

Previous studies have shown that the second-order rate constant k_{cat}/K_m (app) of α-chymotrypsin-catalyzed hydrolysis of specific esters and amides shows a sharp decrease above pH 8 with an apparent dependence on an ionizable acid group with a pK of 8.5–9.0.[1, 2] Further investigations have shown that this decrease in k_{cat}/K_m (app) is due to a drastic increase in the K_m (app) term and more specifically to a decrease in the ability of the enzyme to bind substrates at high pH.[3, 4]

To explain these changes in K_m (app), a pH-dependent equilibrium between two major conformations of the enzyme has been proposed.[3, 4] This equilibrium is thought to be governed by the ionization of the α-amino group of the N-terminal isoleucine 16 that is liberated when chymotrypsinogen is converted into active chymotrypsin by the action of trypsin.[5] Several hypotheses have been formulated to explain the mechanism by which this α-amino group could control the activity and binding ability of the enzyme.[3, 4, 6]

The α-amino group of isoleucine 16 is also present in δ-chymotrypsin, a form of chymotrypsin formed in the trypsin-catalyzed "rapid activation" of chymotrypsinogen.[7] Although a good deal of work has been done in the kinetic properties of α-chymotrypsin at high pH, very little information is available in the literature regarding the kinetic properties of δ-chymotrypsin. Only the kinetics of the hydrolysis of *N*-acetyl-L-tryptophanamide has been previously reported.[4] It is interesting to note in this respect that several experiments such as optical rotation[5] and optical rotatory dispersion,[8] in which important conclusions regarding the behavior of α-chymotrypsin at high pH were obtained, have been done with δ-chymotrypsin. A similarity in the kinetic behavior at high pH between both enzymes has always been assumed.

This paper reports the alkaline pH dependence of the δ-chymotrypsin-catalyzed hydrolysis of the specific substrates *N*-acetyl-L-tryptophan methyl ester and *N*-furylacryloyl-L-tryptophanamide. Kinetic runs with acetylated δ-chymotrypsin were carried out for purposes of comparison. The effect of ionic

strength was also studied. The results are discussed in terms of the present hypothesis for the mechanism of inactivation of α-chymotrypsin at high pH.

Materials and Methods.—All enzymes were obtained from Worthington Biochemical Corp. Salt-free three-times-crystallized chromatographically homogeneous α-chymotrypsin (lot CDS-6602) was used. Quantitative N-terminal group analysis gave 0.85 ± 0.1 mole of isoleucine and 0.65 ± 0.1 mole of alanine per mole of active enzyme. Salt-free three-times-crystallized δ-chymotrypsin (lot CDD-6032) was employed. This enzyme contains 0.85 ± 0.1 mole of isoleucine and 0.0 mole of alanine α-amino terminal residues per mole of active enzyme. Salt-free five-times-crystallized electrophoretically homogeneous chymotrypsinogen A (lot CGC-8CC) and salt-free, two-times-crystallized trypsin (lot TRL-6256) were used.

Acetylated δ-chymotrypsin was prepared by acetylating chymotrypsinogen A with acetic anhydride followed by activation by trypsin according to the procedure of Oppenheimer *et al.*[5] Deacetylation of tyrosyl residues was accomplished by incubating the acetylated enzyme with 0.2 *M* NH_2OH at pH 10.5 for 30 min at 4°C. After purification by chromatography on Sephadex CM-50, the enzyme was shown to contain 0.85 ± 0.1 mole of isoleucine and 0.05 ± 0.03 mole of alanine as α-amino terminal residues per mole of active enzyme; 92% of the ε-amino groups of the lysine residues were shown to be acetylated. Enzyme solutions were freshly prepared and their normality was determined by spectrophotometric titration with *N-trans*-cinnamoylimidazole.[9]

N-Acetyl-L-tryptophan methyl ester was a Cyclo Chemical Co. product (lot 3-4735) and was recrystallized twice from acetonitrile before use (mp 153.0°). *N-trans*-Cinnamoylimidazole was recrystallized four times from hexane (mp 132°) and was a gift from Y. Nakagawa. *N*-2-Furylacryloyl-L-tryptophanamide (lot K5502-2) was obtained from Cyclo Chemical Co. and recrystallized from ethyl acetate–hexane (mp 176–178°). Stock solutions of substrates were made from Eastman nanograde acetonitrile. Buffer solutions of ionic strength 0.1 were prepared from analytical reagent grade materials. Solid KCl was added to prepare buffers of ionic strength 0.5 and 1.0.

The kinetics of hydrolysis was determined with a Cary 14 recording spectrophotometer equipped with a thermostated cell compartment. The hydrolysis of *N*-acetyl-L-tryptophan methyl ester was followed at 300 nm. The hydrolysis of *N*-furylacryloyl-L-tryptophanamide (UV max 305 nm, $\epsilon = 25{,}800$) was followed at 340 nm with 0.1 cm light path cells. The absorbance data were converted into rate data, using $\Delta\epsilon = 740$ as the difference in molar absorptivities between the ester and the acid.[10] The results were analyzed by using a one-run digital computer program based on a least-squares analysis of v/S versus v (Eadie plots).

The pH of each reaction was determined at the beginning and at the end of the reaction by using a Radiometer 4c pH meter with a type B glass electrode. Although most of the runs at high pH reported in this paper were carried out with carbonate buffers, ammonia–ammonium chloride buffers were also used and identical results were obtained.

Quantitative N-terminal group determinations were performed by the method of Sanger.[11] DNP–amino acids were determined spectrophotometrically after separation by thin-layer chromatography as described by Labouesse *et al.*[12] DNP–amino acids obtained from Calbiochem were used as standards.

Results.—The values of K_m (app) and k_{cat} for the δ-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophan methyl ester (ATME) are presented in Table 1 for native δ-chymotrypsin and in Table 2 for acetylated -δ-chymotrypsin. The K_m–pH profiles for the reaction at three different ionic strengths are presented in Figure 1 for both forms of the enzyme. It can be seen that at ionic strength 0.1, K_m (app) for both enzymes increases by a factor of three in the pH region 8 to 10.5–11.0. The curve appears to level off at pH near 11. Increasing the ionic strength to 0.5 or 1.0 causes a remarkable decrease in the K_m (app)

TABLE 1. *Kinetics of the δ-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester.**

pH	Buffer	k_{cat}, sec^{-1}	K_m (app) $\times 10^5$ M
Ionic strength 0.1			
6.96	Phosphate	23.6 ± 0.5	8.8 ± 0.4
7.53	Phosphate	35.4 ± 0.7	8.7 ± 0.6
7.92	Tris-HCl	36.1 ± 1.0	9.6 ± 1.4
8.25	Tris-HCl	41.8 ± 1.0	7.8 ± 0.6
8.50	Tris-HCl	43.4 ± 1.0	9.8 ± 0.4
8.85	Glycine	47.1 ± 1.5	7.6 ± 0.4
9.46	Carbonate	45.5 ± 0.5	13.6 ± 0.5
9.51	Carbonate	45.2 ± 2.5	14.8 ± 0.5
9.70	Glycine	46.6 ± 3.5	16.5 ± 0.6
10.02	Carbonate	41.2 ± 0.8	19.2 ± 0.7
10.20	Carbonate	37.3 ± 0.8	21.3 ± 0.8
10.35	Glycine	48.2 ± 1.7	23.2 ± 1.0
10.67	Carbonate	41.2 ± 1.5	26.9 ± 1.2
10.78	Carbonate	40.5 ± 1.5	27.0 ± 1.3
10.97	Corbonate	36.4 ± 1.6	26.9 ± 0.6
11.10	Carbonate	38.1 ± 1.0	32.4 ± 0.8
11.15	Carbonate	34.5 ± 1.5	31.2 ± 1.8
Ionic strength 0.5			
6.72	Phosphate	20.3 ± 0.5	7.5 ± 0.3
7.24	Phosphate	33.0 ± 1.5	6.6 ± 0.5
8.05	Tris-HCl	43.0 ± 1.0	5.1 ± 0.6
8.74	Tris-HCl	46.3 ± 1.0	5.8 ± 0.3
8.98	Glycine	48.0 ± 1.0	4.8 ± 0.4
9.22	Carbonate	47.5 ± 0.9	8.5 ± 0.4
9.41	Carbonate	44.7 ± 1.1	8.0 ± 0.5
9.57	Carbonate	44.9 ± 2.0	9.0 ± 0.6
10.03	Carbonate	38.6 ± 1.5	10.3 ± 2.6
10.41	Carbonate	34.8 ± 2.0	11.8 ± 0.6
10.48	Carbonate	33.2 ± 1.8	10.6 ± 0.7
11.02	Carbonate	29.5 ± 1.5	12.0 ± 1.2
Ionic strength 1.0			
7.12	Phosphate	30.3 ± 0.5	5.9 ± 0.5
7.98	Phosphate	43.4 ± 1.0	5.3 ± 0.6
8.35	Tris-HCl	45.6 ± 1.0	4.3 ± 0.5
8.52	Tris-HCl	45.8 ± 0.5	4.9 ± 0.8
9.02	Carbonate	35.3 ± 1.0	4.4 ± 0.4
9.29	Carbonate	44.1 ± 1.5	6.2 ± 0.7
9.38	Carbonate	43.1 ± 0.5	6.9 ± 0.3
9.79	Carbonate	45.0 ± 0.5	8.1 ± 0.2
10.18	Carbonate	29.4 ± 1.0	9.0 ± 0.5
10.32	Carbonate	35.7 ± 1.5	8.0 ± 0.8
10.67	Carbonate	32.0 ± 3.5	7.8 ± 1.2
10.86	Carbonate	25.6 ± 1.5	7.6 ± 1.0

* 1.6% (v/v) acetonitrile–water at 25 ± 0.2°C. $[E_0] = 1$ to 3×10^{-7} M. $[S_0] = 6$ to 8×10^{-4} M.

values, particularly in the high pH region. The value of K_m (app) at pH 11 is less than two times the value at pH 8. The profiles corresponding to ionic strength 0.5 and 1.0 definitely level off and the K_m (app) values are independent of pH above 9.8.

When the data were plotted according to the method of Dixon,[13] approximate values for the pK (app) for the ionizing group involved were obtained. A pK_E (app) value of 9.25 was obtained for the pH dependence of K_m at the three different ionic strengths. This pK is perturbed by substrate binding to pK_{ES} (app)

TABLE 2. *Kinetics of the acetylated-δ-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-tryptophan methyl ester.**

pH	Buffer	k_{cat}, sec^{-1}	K_m (app) $\times 10^5 M$
Ionic strength 0.1			
7.09	Phosphate	28.4 ± 0.7	6.6 ± 0.7
7.61	Phosphate	41.4 ± 1.5	8.4 ± 1.0
7.92	Tris-HCl	43.2 ± 0.7	6.6 ± 0.5
8.54	Tris-HCl	44.7 ± 1.0	6.8 ± 0.4
9.04	Glycine	49.7 ± 1.5	8.9 ± 2.9
9.29	Carbonate	45.2 ± 0.5	11.8 ± 0.7
9.57	Carbonate	45.2 ± 0.5	16.5 ± 0.5
9.71	Carbonate	40.6 ± 1.2	19.5 ± 1.5
9.93	Carbonate	31.2 ± 1.5	18.6 ± 2.0
10.33	Carbonate	37.3 ± 3.0	25.0 ± 2.8
10.65	Carbonate	36.0 ± 5.0	24.5 ± 5.0
10.85	Carbonate	32.4 ± 2.0	26.5 ± 2.0
11.15	Carbonate	24.8 ± 1.0	22.9 ± 2.6
11.25	Carbonate	24.1 ± 2.0	22.6 ± 4.2
Ionic strength 0.5			
6.74	Phosphate	33.6 ± 1.0	6.5 ± 0.2
7.25	Phosphate	46.5 ± 2.5	5.6 ± 0.7
8.05	Tris-HCl	53.6 ± 1.0	6.4 ± 0.1
8.69	Tris-HCl	52.5 ± 1.5	6.2 ± 0.4
8.98	Glycine	56.1 ± 1.5	6.0 ± 0.4
9.30	Carbonate	47.1 ± 2.0	9.5 ± 0.6
9.54	Carbonate	45.1 ± 1.8	10.9 ± 1.5
10.09	Carbonate	34.9 ± 2.0	11.9 ± 2.0
10.42	Carbonate	28.9 ± 1.0	12.2 ± 1.0
10.64	Carbonate	23.8 ± 3.8	11.4 ± 1.5
11.11	Carbonate	28.0 ± 2.0	11.8 ± 0.8
Ionic strength 1.0			
7.16	Phosphate	38.2 ± 2.0	5.8 ± 1.4
7.87	Phosphate	48.9 ± 1.0	5.4 ± 0.5
8.13	Tris-HCl	48.5 ± 1.0	5.0 ± 0.5
8.56	Tris-HCl	47.6 ± 1.0	3.9 ± 1.5
8.77	Glycine	50.4 ± 1.2	4.0 ± 0.8
9.32	Carbonate	45.5 ± 1.5	6.0 ± 0.8
9.50	Carbonate	44.7 ± 2.0	6.8 ± 0.7
9.71	Carbonate	45.0 ± 0.5	8.8 ± 0.5
10.01	Carbonate	41.9 ± 0.5	9.2 ± 0.2
10.32	Carbonate	35.3 ± 3.5	8.7 ± 1.8
10.58	Carbonate	30.6 ± 2.0	8.8 ± 1.1
11.10	Carbonate	25.6 ± 1.0	8.8 ± 0.7

* 1.6% (v/v) acetonitrile–water at 25 ± 0.2°C. $[E_0] = 1$ to $3 \times 10^{-7} M$. $[S_0] = 6$ to $8 \times 10^{-4} M$.

values of 9.75, 9.55, and 9.50 for the reaction at ionic strengths 0.1, 0.5, and 1.0, respectively. The results indicate that the main effect produced by increasing the ionic strength in the δ-chymotrypsin-catalyzed hydrolysis of ATME is a shift in the pK_{ES} (app) to a lower value.

The results so far obtained indicate that the behavior of δ-chymotrypsin in its reaction with ATME at high pH contrasts markedly with the well-known behavior of α-chymotrypsin with the same specific substrate.[1, 3] In Figure 2 the pH dependence of K_m (app) for the reaction of δ-chymotrypsin with ATME is compared with that of α-chymotrypsin at the same ionic strength. It can be seen that the profiles differ significantly. The K_m (app) values for the α-chymotrypsin-catalyzed reaction increase sharply above pH 8.8 in agreement

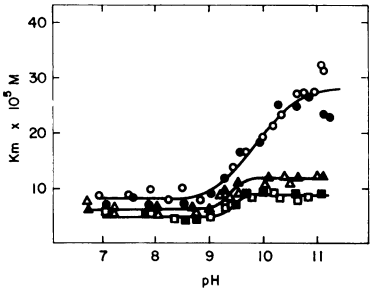

FIG. 1 (*above*).—pH dependence of K_m (app) for the δ-chymotrypsin-catalyzed hydrolysis of ATME. ○, δ-Chymotrypsin at ionic strength 0.1; ●, acetylated δ-chymotrypsin at ionic strength 0.1; △, δ-chymotrypsin at ionic strength 0.5; ▲, acetylated δ-chymotrypsin at ionic strength 0.5; □, δ-chymotrypsin at ionic strength 1.0; ■, acetylated δ-chymotrypsin at ionic strength 1.0; For buffer composition see Tables 1 and 2. Experimental conditions were described in *Materials and Methods*.

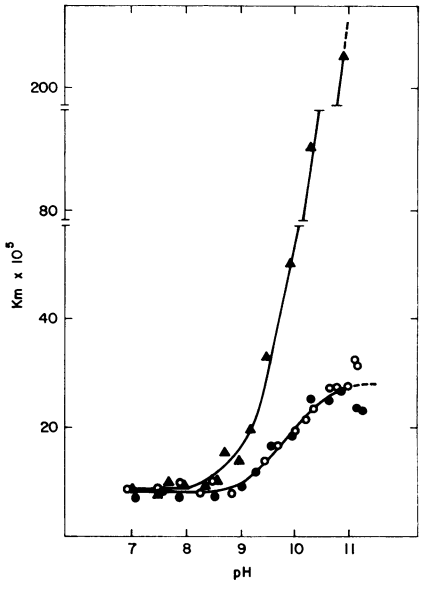

FIG. 2 (*right*).—pH dependence of K_m (app) for the α-chymotrypsin and δ-chymotrypsin-catalyzed hydrolyses of ATME at ionic strength 0.1. △, α-Chymotrypsin; ○, δ-chymotrypsin; ●, acetylated δ-chymotrypsin. For experimental conditions see *Materials and Methods*.

with previous reports.[3] The K_m (app) values for the δ-chymotrypsin-catalyzed reaction only increase by a factor of three from pH 8 to pH 11. For α-chymotrypsin a pK_E (app) value of 9.0 was obtained.

The results obtained for the δ-chymotrypsin-catalyzed hydrolysis of *N*-furylacryloyl-L-tryptophanamide (FATA) are presented in Table 3 and Figure 3. Although the K_m (app) values obtained in this research with FATA are somewhat higher, the K_m (app)–pH profile is quite similar to that reported by Himoe *et al.* for the δ-chymotrypsin-catalyzed hydrolysis of *N*-acetyl-L-tryptophanamide (ATA).[4] It can be seen from Figure 3 that K_m (app) increases only about three times from pH 8 to pH 10.5–11.0. Above this pH the values of K_m (app) seem to become independent of the pH. The same is true for the δ-chymotrypsin-catalyzed hydrolysis of ATA.[4] From the results given in Table 3, values of pK_E (app), 9.5, and pK_{ES} (app), 10.0, were obtained. The same behavior, within the experimental error, was obtained at ionic strengths 0.1 and 0.5. Again it is possible to note the difference between the K_m (app)–pH profile reported here for the δ-chymotrypsin reaction with FATA and the K_m (app)–pH profile previously reported for the reaction of α-chymotrypsin with ATA,[3, 4] where K_m (app) increases progressively above pH 8.8 to much higher values, very similar to the result obtained with ester substrates.[3]

In order to compare the behavior of both enzymes at high pH, we have measured the second-order rate constant k_{cat}/K_m (app) for the reaction of δ- and α-chymotrypsin with FATA at pH 11.0. A value of 8.00 M^{-1} sec^{-1} was obtained for the reaction with δ-chymotrypsin and 0.10 M^{-1} sec^{-1} for the reaction with α-chymotrypsin. This indicates that at pH 11.0 δ-chymotrypsin is approximately 80 times more active than α-chymotrypsin toward FATA.

TABLE 3. *Kinetics of δ-chymotrypsin-catalyzed hydrolysis of N-2-furylacryloyl-L-tryptophanamide.**

pH	Buffer	$k_{cat} \times 10^2$ sec^{-1}	K_m (app) $\times 10^3$ M
Ionic strength 0.1			
7.45	Phosphate	15.1 ± 2.0	5.0 ± 1.0
8.03	Tris-HCl	19.1 ± 2.0	6.0 ± 1.2
8.30	Tris-HCl	19.8 ± 3.0	8.1 ± 2.0
8.45	Tris-HCl	22.0 ± 2.3	8.0 ± 1.5
8.70	Tris-HCl	14.5 ± 4.5	4.2 ± 0.8
9.05	Glycine	20.0 ± 2.0	4.5 ± 1.2
9.25	Carbonate	17.5 ± 2.5	7.0 ± 3.0
9.65	Glycine	19.6 ± 0.8	11.0 ± 1.0
10.00	Carbonate	19.1 ± 2.0	14.8 ± 1.8
10.40	Carbonate	15.0 ± 2.0	18.0 ± 2.0
10.80	Carbonate	12.0 ± 2.0	15.0 ± 3.0
Ionic strength 0.5			
8.17	Tris-HCl	23.4 ± 2.6	9.1 ± 2.4
9.04	Carbonate	18.1 ± 1.5	5.5 ± 1.0
9.30	Carbonate	26.2 ± 2.5	7.3 ± 3.3
9.80	Carbonate	12.0 ± 2.8	10.3 ± 2.1
10.01	Carbonate	12.5 ± 2.5	17.8 ± 1.4
10.80	Carbonate	12.3 ± 4.2	23.7 ± 6.1

* 8.4% (v/v) acetonitrile–water at 25 ± 0.2°C. $[E_0] = 5$ to 6×10^{-5} M. $[S_0] = 1.2$ to 1.5×10^{-2} M.

Discussion.—Our data indicate that there is a profound difference in the alkaline pH dependence of K_m (app) for ATME and FATA between δ-chymotrypsin and α-chymotrypsin. This finding, together with that previously reported for the hydrolysis of ATA,[4] suggests that this is a general phenomenon, at least for specific substrates. The fact that this difference is observed with specific amide substrates, where K_m is equal to K_s,[14, 15] indicates that the difference between the two enzymes lies in a different pH dependence of K_s, the dissociation constant for the ES complex.

Increase of the ionic strength results in a decrease in K_m (app) for ATME. This effect is more pronounced in the alkaline pH region. It has been shown that for specific ester substrates K_m is equal to $k_3K_s/(k_2 + k_3)$. Our results with ATME indicate the k_3 does not change with ionic strength. Kinetic experiments carried out in this laboratory with *N*-acetyl-L-valine methyl ester show a decrease in K_m and no change in k_2.[16] So the changes in K_m with ionic strength observed for the reaction of δ-chymotrypsin with ATME can be attributed to a decrease in K_s rather than an increase in k_2 or decrease in k_3.[17]

Our results show that an ionizing group with a pK (app) of 9.25 is involved in the binding of ATME to δ-chymotrypsin. This pK is shifted to a higher value

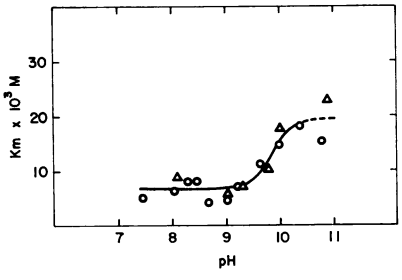

FIG. 3.—pH dependence of K_m (app) for the δ-chymotrypsin-catalyzed hydrolysis of FATA. O, ionic strength 0.1; Δ, ionic strength 0.5. Buffer composition is in Table 3. Experimental conditions as described in *Materials and Methods.*

upon binding of the substrate to the enzyme. This shift is ionic-strength-dependent and the difference between pK_E (app) and pK_{ES} (app) at ionic strength 0.5 is only 0.3, indicating that the binding of the substrate to δ-chymotrypsin at this ionic strength exhibits a rather small dependence on pH. The pK (app) values obtained in this research are in qualitative agreement with the values determined by Hess and co-workers from substrate-induced proton uptake experiments with ATA.[18]

A pH-dependent equilibrium between active and inactive conformations of the enzyme has been proposed to explain the drastic decrease in the binding ability of α-chymotrypsin at alkaline pH.[3, 4] This equilibrium is assumed to be governed by the ionization of the α-amino group of isoleucine 16.[5] A pH-dependent intramolecular competitive inhibition has been proposed to explain the mechanism of action of this residue.[3] An alternative explanation has been developed by Sigler *et al.* in light of the X-ray diffraction studies on the structure of crystals of tosyl-α-chymotrypsin.[6] They have proposed that the active conformation of the enzyme in solution is stabilized by a salt bridge between the ammonium group of isoleucine 16 and a carboxylate group of aspartic 194 in a region of the enzyme of low dielectric constant.

It is quite likely that the group involved in the K_m (app)–pH transitions reported here for δ-chymotrypsin is the α-amino group of isoleucine 16.[3, 5] Our results indicate, however, that the state of ionization of this group in δ-chymotrypsin is not crucial for the binding ability of the enzyme, especially at ionic strength 0.5 or higher. Thus, our results do not seem to support the hypothesis of Sigler *et al.*[6] δ-Chymotrypsin binds substrate, and it is active in conditions in which salt bridge between isoleucine 16 and aspartic acid 194 should not exist (pH above 11 and ionic strength 1.0). This suggests that the formation of active δ-chymotrypsin from chymotrypsinogen as well as the inactivation of δ-chymotrypsin by acetylation of the α-amino group of isoleucine 16 may not be related with a formation or disruption of an essential salt bridge.[6, 8]

The still unknown mechanism of inactivation of α-chymotrypsin at high pH may be tentatively explained by considering the fact that α-chymotrypsin differs from δ-chymotrypsin by the presence of two new terminal residues, the carboxy terminal tyrosine 146 and the amino terminal alanine 149.[19] Both residues have groups that ionize at alkaline pH and are chain termini. Therefore, we can expect that they are fairly mobile and have increased possibilities of interaction with other groups of the enzyme.[20] One explanation is that the ionization of one or both of these groups in α-chymotrypsin produces a change in the relative position of the residues responsible for binding. This may increase the inhibitory or disruptive effect of the deprotonation of isoleucine 16 relative to the effect of this residue in δ-chymotrypsin. A second and more attractive possibility involves a more direct interaction between alanine 149 or tyrosine 146, or both residues, with the groups involved in the binding and activity of the enzyme. Experimental research using these working hypotheses is in progress in our laboratory.

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[1] Bender, M. L., G. E. Clement, F. J. Kézdy, and H. d'A. Heck, *J Am. Chem. Soc.*, **86**, 3680 (1964).

[2] Himoe, A., and G. P. Hess, *Biochem. Biophys. Res. Commun.*, **23**, 234 (1966).

[3] Bender, M. L., M. J. Gibian, and D. J. Whelan, these PROCEEDINGS, **56**, 833 (1966).

[4] Himoe, A., P. C. Parks, and G. P. Hess, *J. Biol. Chem.*, **242**, 919 (1967).

[5] Oppenheimer, H. L., B. Labouesse, and G. P. Hess, *J. Biol. Chem.*, **241**, 2720 (1966).

[6] Sigler, P. B., D. M. Blow, B. W. Matthews, and R. Henderson, *J. Mol. Biol.*, **35**, 143 (1968).

[7] Jacobsen, C. F., *Compt. Rend. Trav. Lab. Carlsberg*, Serie Chimique, **25**, 325 (1947).

[8] McConn, J., G. D. Fasman, and G. P. Hess, *J. Mol. Biol.*, **39**, 551 (1969).

[9] Schonbaum, G. R., B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).

[10] Although, because of the lack of solubility, the kinetic runs with FATA were done in the critical conditions: $[S]/K_m = 1$ to 4, the values of k_{cat} and K_m extrapolated from Eadie plots agreed well with the value of k_{cat}/K_m obtained by first-order kinetics, under conditions where $[S] < K_m$.

[11] Sanger, F., *Biochem. J.*, **39**, 507 (1945).

[12] Labouesse, J., and M. Gervais, *European J. Biochem.*, **2**, 215 (1967).

[13] Dixon, M., *Biochem. J.* **55**, 161 (1961).

[14] Zerner, B., and M. L. Bender, *J. Am. Chem. Soc.*, **86**, 3669 (1964).

[15] Brandt, K. G., A. Himoe, and G. P. Hess, *J. Biol. Chem.*, **242**, 3973 (1967).

[16] Payton, C., unpublished experiments from this laboratory.

[17] Experimental work is currently in progress in our laboratory to verify this conclusion.

[18] McConn, J., E. Ku, C. Odell, G. Cerlinski, and G. P. Hess, *Science*, **161**, 274 (1968).

[19] Desnuelle, P., *Enzymes*, **4**, 93 (1960).

[20] It has been shown that both residues contribute to intermolecular interactions in crystalline α-chymotrypsin.[6] Gladner and Neurath have shown that tyrosine 146 is sufficiently exposed to be rapidly attacked by carboxypeptidase.[21] The same residue has been reported to be the most rapidly iodinated residue in α-chymotrypsin.[22]

[21] Gladner, J. A., and H. Neurath, *J. Biol. Chem.*, **206**, 911 (1954).

[22] Glazer, A. S., and F. Sanger, *Biochem. J.*, **90**, 92 (1964).